Particularities of Real-Time Polymerase Chain Reaction Technique (RT-PCR) in the Monitoring of Chronic Myeloid Leukemia Patients – A Brief Overview

Kinga Tatar, Hortensia Ionita

Department of Hematology, University of Medicine and Pharmacy “Victor Babeș”, Timișoara

ABSTRACT Chronic myeloid leukemia (CML) is a myeloproliferative disorder, characterized by a specific chromosomal aberration, the Philadelphia [Ph] chromosome. The Ph chromosome is the result of a reciprocal translocation between the long arms of chromosomes 9 and 22, t (9;22)(q34;q11). The molecular consequence of this translocation is a novel fusion gene, BCR-ABL, which encodes a constitutively active tyrosine kinase, implicated in pathophysiology and development of CML [1,2]. Imatinib mesylate currently the golden standard for front line treatment of CML, is a selective inhibitor of the BCR-ABL tyrosine kinase activity. Responses to imatinib occur at hematologic, cytogenetic and molecular levels. IM therapy now allows the majority of patients with CML to reach CCyR - a confirmed good prognostic indicator [3]. In different studies, 87% of patients in chronic, 17% of patients in accelerated, and 7% of patients in blast phases reached the important clinical aim of Ph negativity [3-5]. Ph chromosome status is usually assessed by classical bone marrow cytogenetics and this method has the limitation of poor sensitivity. Patients who reveal 10^12 leukemic cells at the time of diagnosis, in CCyR may still have as many as 10^10 leukemic cells in their body. Once a patient has achieved CCyR, monitoring of residual cells is usually performed by estimating the BCR-ABL transcripts on a molecular level using quantitative real-time polymerase chain reaction (QRT-PCR). A 2-log reduction in BCR-ABL transcripts correlates with Ph negativity in CCyR. Patients achieving a 3-log reduction in BCR-ABL transcripts are defined as having a major molecular response (MMR), a surrogate marker closely correlating with the probability of disease free survival [8]. Patients failing to achieve this 3-log response, at any time during therapy, had significantly shorter progression-free survival [6]. After a 5 to 6 log reduction, BCR-ABL transcripts can no longer be detected by QRT-PCR and patients are designated as having complete molecular remission (CMR). But even with the most sensitive QRT-PCR assay, CMR is consistent with the persistence in the patient’s body of up to 10^6 or 10^7 leukemic cells [7]. Due to the proliferation of such residual cells a significant fraction of those patients who have responded on a deep molecular level, lose this response and progress to advanced phase disease. The clinical advantage of the extremely sensitive method of QRT-PCR is to be alerted by rising transcript levels at a very early time point, usually weeks or even months before the onset of clinical symptoms, allowing early therapeutic intervention with a beneficial impact on survival.

KEY WORDS BCR-ABL mRNA, control gene, Lightcycler, TaqMan, major molecular response, minimal residual disease

Introduction

RT-PCR in CML—some technical considerations

RT-PCR technique has evolved over the years from qualitative PCR for assessing the presence or absence of BCR-ABL transcripts after allogeneic stem cell transplantation to nested PCR and finally to real time PCR techniques [15].

In CML the real-time PCR technique combines with reverse transcription PCR in order to quantify the level of BCR-ABL RNA m levels. BCR-ABL mRNA is transcribed in complementary DNA using reverse transcriptase, followed by the amplification the obtained DNA by the polymerization reaction.

This technique is using eight TaqMan (Byosistem Foster City, CA), or LightCycler (Roche Diagnostics, Indianapolis) methodologies.

The TaqMan Real-time PCR measures accumulation of a product via the fluorophore during the exponential stages of the PCR, rather than at the end point as in conventional PCR [11]. The exponential increase of the product is used to determine the threshold cycle, CT, i.e. the number of PCR cycles at which a significant exponential increase in fluorescence is detected, and which is directly correlated with the number of copies of DNA template present in the reaction. The set up of the reaction is very similar to a conventional PCR, but is carried out in a real-time thermal cycler that allows measurement of fluorescent molecules in the PCR tubes.

LightCycler methodology is using a platform called LightCycler—the only technology currently accredited for routine molecular diagnosis; all other technologies are accredited only for research purposes. The used quantifying method is based on hybridization probes using the Free Resonance Energy Transfer (FRET).
Figure 1 - Approximate relationship between response, the putative number of leukemic cells and the level of BCR-ABL transcripts.[8]

The major advantages introduced are [13]:
- the kinetics of the PCR is followed during amplification, rather than at the end-point, eliminating the need for co-amplification of a competitor
- the fluorescence based technology enhances the reproducibility
- increasing the dynamic of the reaction
- the real-time detection eliminates the need for post-PCR manipulation

As previously described, the existing techniques vary with respect of the type of device, the location of the probes, the type of real-time reaction and the control gene.

These differences translate from clinical point of view in few downsides:
- Large variability of determinations (up to 0.5 log or 2 – 5 %)
- False negative results in laboratories using a low sensitivity QRT-PCR technique
- Lack of international standardization leads to difficulties in interpreting results by clinicians.

In the attempt of overcoming this downsides, in 2003 took place a collaborative action called Europe Against Cancer(EAC), including 26 laboratories from 10 countries [14]. The scope of this program was to establish standardized protocols for fusion gene quantification by TaqMan technique. It was proposed to be used a common forward primer placed on the 13th exon of BCR gene, and a reverse primer located on the exon 2 of gene ABL, for amplification of both transcripts (b2a2, b3a2).[15]

Figure 2. Taq Man assay [19]

Figure 3. Hybridization probes format-overview [20]

Using standardized protocols, EAC identified the most appropriate control gene to be amplified in parallel with the sample.[14] The role of this control gene is to correct the qualitative and quantitative variations of the RNA and to appreciate the sensitivity of each determination.

From the initial number of 14 candidate genes, based on the absence of pseudogenes, stability and gene expression there were 3 genes selected: Abelson (Abl), beta-2-microglobulin (B2M) and betaglucuronidase (GUS). Although all 3 genes had a stable expression in the studied samples, it was established that only ABL gene had similar expressions in samples from both healthy and leukemic subjects, and it was proposed as control gene.

Figure 4. Schematic diagram of the BCR-ABL FG transcript covered by the RQ-PCR primer and probe set [15]

Reporting of results

Currently there are different ways of reporting RT-qPCR results. One of this is to report the number of bcr-abl copies per microgram of RNA, but this approach has some disadvantages: the measurement of RNA concentration is not exact, and the quality and efficacy of the reverse transcription is not taken in to account. Another approach is to express the results as a ratio of the
number of BCR-ABL copies and the copies of the control gene. According to this approach the equal number of BCR-ABL copies and control gene at diagnostic represents 100%. In the IRIS study it was used for the first time the concept of logarithmic reduction from a standardized baseline of untreated patients. Achievement of a 3 log reduction or more from the standardized baseline was considered as a Major Molecular Response and achieving this was strongly associated with a better progression free survival. The baseline standard was established by measuring the BCR-ABL/ABL ratio in 30 CML patients before treatment initiation. The same 30 samples were processed in 3 different laboratories. This approach has several advantages: makes possible the expression of results on a common international scale, once the laboratory has established the standard baseline and furthermore it is possible to evaluate the level of response for a patient without actually knowing the pretreatment level of BCR-ABL. However this method has the disadvantage that in order to establish a save level for the minimal residual disease it is necessary to exchange a large number of samples between several labs. In order to overcome this disadvantage it was proposed that the Standardized Baseline from IRIS study to be considered as 100% and the 3 log reduction (corresponding to MMR) to be established at 0.1% [16]. In order to express results on an international scale, each laboratory has to establish a conversion factor by using a series of samples with known value. By using this CF, the BCR-ABL value obtained in a certain laboratory (BCR-ABL L) can be expressed on the International Scale (BCR-ABL IS) using the formula:

\[ \text{BCR-ABL IS} = \frac{\text{BCR-ABL L} \times \text{CF}}{\text{MMR IS}/\text{MMR Eq}} \]

The Conversion Factor (CF) is calculated according to the bellow formula, where MMR IS the value of BCR-ABL corresponding to the MMR in the IRIS study and MMR Eq represents the value of BCR-ABL L corresponding to RMM as defined in the IRIS study.

However there is a need for additional efforts for standardizing this technique by using standardized reference samples, an essential step in obtaining comparable results in across different laboratories.

From the laboratory bench work to the clinical decision

Achieving a 2 log reduction in the transcript level at the moment of complete cytogenetic or a 3 log reduction at any moment thereafter represents an independent prognostic factor for the progression free survival [6].

Molecular studies performed in the IRIS trial have demonstrated for the first time that the level of minimal residual disease at 12 months expressed as log reduction from the baseline is significantly associated with the event free survival and risk of progression in newly diagnosed patients who achieved a CCR under imatinib treatment. This threshold of 3 or more BCR-ABL log reduction identified a subset of patients with an extremely favorable prognostic, with only sporadic loss of responses and very low risk for progression. These results were confirmed also on the longer follow-ups of the IRIS trial. Another study where cytogenetic examination was scheduled at one year, demonstrated that a rapid fall in the BCR-ABL mRNA level can predict the cytogenetic response, having an important prognostic value. Based on the increasing evidence regarding the impact of BCR-ABL reduction on the prognostic of imatinib treated patients the European LeukemiaNet introduced this parameter in the operational definition of the optimal response to imatinib therapy.

The presence of MMR at 18 months is considered as optimal response and according to ELN the significance is long term outcome for patients not achieving this response on imatinib treatment might not be favorable [8].

In a more recent study performed at Hammersmith Institution it was performed the outcome analysis for 224 chronic –phase CML patients in an attempt to assess for the extent to which the ELN recommendation can be validated in clinical practice.

According to this study patients in CcyR who failed to achieve MMR at 12 or 18 months were more likely to lose their CcyR than patients who
achieved MMR: 23.6% vs. 2.6% and 24.6% vs ), although there was no significant impact on the PFS and OS [18].

**Definition of molecular responses**

As previously mentioned there were many controversies regarding the definition and expression of major molecular response, at this moment most working parties recommend to define MMR as a decrease of transcript level to 0.1% from the baseline standard level. The definition of complete molecular response as an undetectable transcript corresponds to a 4-5 log reduction, but is subject to the sensibility variations of the technique used. For this reason whenever a CMR is reported it is recommended that the sensibility of the technique to be also communicated and this result should be confirmed by nested PCR.

**Recommended frequency for monitoring:**

After the initiation of a TKI treatment it is recommended to monitor the BCR-ABL level once every 3 months, before and after a complete cytogenetic response is achieved. It is clinically relevant to characterize the response as: [13]:

- descending transcript
- undetectable transcript
- stable transcript level (plateau)
- increasing transcript level

Regarding the definition and significance of the increasing transcript level, there are still ongoing controversies: the Adelaide group found a statistical significance between the 2 fold increase in BCR-ABL transcript level and the emergence of mutations. The technique used in this study was extremely sensitive, so in case of less sensitive settings it is recommended to perform mutational analysis in case of a 5-5 fold increase of the transcript level, confirmed by successive determinations. In practice the BCR-ABL level increase is more frequently associated with kinase domain mutations in patients who never achieved a major molecular response.

**Conclusion**

The prognostic of CML patients considerably improved over the past years due to highly efficient therapeutic options. In consequence the adequate monitoring of these patients is becoming more and more important. In the past years several studies in CML patients propose as efficacy endpoint the achievement of major or complete molecular response in patients with complete cytogenetic response. However the long term prognostic significance of these molecular parameters is still to be determined. Although the standardization of this technique still needs some fine tunings, the role of molecular monitoring in CML patients is clearly established. The use of this technique makes possible the definition of responses as optimal/ suboptimal and moreover it identifies a category of patients with extremely favorable prognosis, it can be an early predictor for achieving the CCR, but in the same time for relapse, disease progression or emergence of kinase domain mutation. In the era of tyrosine kinase inhibitors, this technique should be available to all hematologists in order to provide an optimal standard of care for CML patients.

**References**


15. Gabert, E Beillard1,VHJ van der Velden, W Bi, D Grimwade et al, Standardisation and quality control studies of "real-time " quantitative reverse transcriptase polymerase chain reaction (RQ-PCR)-a Europe against cancer program, Leukemia 2003; 17: 2318-2357.


Correspondence Adress: Kinga Tatar MD , Department of Hematology, University of Medicine and Pharmacy “Victor Babeş”, City Hospital Timisoara, Gheorghe Dima Street, No.5Timisoara , tatarkinga@yahoo.com

Department of Hematology,City Hospital Timisoara, Gheorghe Dima Street, No.5